Chapter 2

Head and Neck Cancer: Epidemiology and Role of MicroRNAs

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Abstract

Head and neck cancer (HNC) is referred to the cancers of aerodigestive tract covering number of structures *viz*, oral and nasal cavity, paranasal sinuses, lips, salivary glands, oropharynx, hypopharynx, pharynx, larynx, and local lymph nodes. It is the sixth most common cancer in the world. MicroRNAs (miRNAs) are small single-stranded noncoding RNAs (ncRNAs) of about 19–25 nucleotides. These miRNAs have been reported to influence number of biological activities, i.e., gene regulation, differentiation, organ formation, cell death, cell proliferation, and stress responses. The first ever study involving miRNAs in HNC was published in 2005. Since then, association between dysregulation of miRNAs and head and neck tumorigenesis has been documented by a number of researchers. This chapter has covered a comprehensive state of the art literature review of the recent studies about the role of miRNAs in HNC including oral squamous cell carcinoma (OSCC) and human nasopharyngeal carcinoma. Despite significant improvement in multimodal treatment, the prognosis of advanced HNC is quite poor. Recent studies are promising regarding the potential role of miRNAs as prognostic indicators. Recently, some miRNAs have been discovered as important diagnostic biomarkers. In fact, miRNAs are found circulated stably in different body fluids, i.e., urine, blood, saliva, as well as in breath. Hence, these miRNAs can be assessed easily with noninvasive methods. miRNAs are the key therapeutic targets in addition to their prognostic and diagnostic value. Use of synthetically designed "miRNAs sponges," miR mimics (agomiRs), miR antagonists ("antagomiRs"), and miR inhibitors (antimiRNAs oligonucleotides) is an innovative strategy to modulate oncogenic and tumor-suppressive pathways. Our understanding of miRNAs involvement in HNC is in its infancy. The discovery of miRNAs heralds a complete new paradigm in the understanding of exact molecular pathways involved in HNC development. More detailed studies are required for better understanding and therapeutic targets to treat HNC.

Keywords: biomarker, gene regulation, head and neck cancer, microRNAs, nasopharyngeal carcinoma, noncoding RNAs, oral squamous cell carcinoma (OSCC), therapeutic targets

1. Head and neck cancer epidemiology

Head and neck cancer (HNC) is referred to the cancers of aerodigestive tract covering number of structures *viz*, oral and nasal cavity, paranasal sinuses, lips, salivary glands, oropharynx, hypopharynx, pharynx, larynx, and local lymph nodes [1]. It is the sixth most common cancer in the world with more than 500,000 cases reported annually [2, 3]. HNC has found to be more prevalent among males than females [3]. More than 90% of all the reported cases of HNC are squamous cell carcinomas (HNSCCs) which usually arise from mucosal lining in these sites [1]. Among these cases, 80–90% cases were found to be associated with prolonged usage of alcohol and tobacco [4].

One of the frequently diagnosed malignancies, globally, is head and neck cancer. Frequent relapses and distant metastasis have been observed in patients with advanced disease stages, resulted in an endurance rate of 5 years in almost 60% patients despite of substantial advances in multimodality rehabilitation [5]. Mucosal malignancy of head and neck is a potent melanoma with poor prognosis. The nasal pit, paranasal sinuses, and oral cavity are the most widely recognized areas. Survival rates of 1, 3, and 5 years were 63, 30, and 20%, respectively, around 2000–2007. All interpretations suggested that cigarette smoking is hazardous. Clinical marks and clues are typically nonspecific for it. Surgery is regarded as the backbone of treatment for most mucosal melanomas of the head and neck supplemented with radiotherapy [6]. The relationship amongst alcohol consumption and head and neck disease is quite clear and the outcomes reliably demonstrated an expanded head and neck tumor chance related with alcohol drinking. Bagnardi et al. reported a positive relationship in between alcohol drinking and head and neck cancer risk [7]. In Western nations, around 39% of head and neck malignancy can be ascribed to alcohol utilization (4% for only alcohol drinking and 35% for the joint impact of alcohol and tobacco) [8]. Head and neck squamous cell carcinomas (HNSCCs) cause more than 300,000 deaths globally every year. Locoregional and removed recurrences are more important prognostic indicators and acknowledged surrogate markers of patients' survival. No legitimate biomarker and rescue treatment exist to recognize and treat patients at high-danger of recurrence [9]. Despite of reduction in smoking and alcohol utilization, the rate of oropharyngeal squamous cell carcinoma (OPSCC) is rising. It refers to human papilloma virus (HPV) infection contamination [10]. HPV is an entrenched prognostic marker for OPSCC [11]. Oropharyngeal tumors are firmly connected with HPV-positivity [12]. Oral tumor constitutes the dominant part of head and neck diseases, which is the fifth most basic malignancy around the world, representing 984,430 cases in 2012. During 2000 and 2010, there were 1916 instances of OSCC in New Zealand with a male to female proportion of 1.85:1, and an age-institutionalized rate of 42 for every 1,000,000 people [13]. Liquor utilization, trailed by tobacco, is considered the most common hazard in New Zealand. Given the high pervasiveness of these two hazardous elements and their synergistic impact, it is vital for specialists to boost smoking cessation and limited liquor consumption. More research should be conducted to confirm use of tobacco and water-pipe smoking in New Zealand, particularly because of changing demography and increments in transient populaces. UV radiation is an additionally imperative hazard element [13]. Laryngeal squamous cell carcinoma (LSCC), being a

potent threat, is amongst the most regularly analyzed malignant sorts of head and neck SCC around the world. Rates of LSCC have been estimated to escalate recently [14]. Salivary gland pleomorphic adenoma (SGPA) is also one of the most widely recognized types of salivary organ tumor. In China, particularly in the South, nasopharyngeal cancer (NPC) is another widely recognized threatening tumors and hence remained unregistered even by National cancer registries, since little is known about its epidemiology [15]. The occurrence represented around 40% of the world's new cases as indicated by the World Health Organization's GLOBOCAN revealed information of 2012 [16]. Individuals with a family history of NPC have a generously higher danger of NPC [17]. NPC's mortality indicates marked distinction between endemic (highly vulnerable territories), where nonkeratinizing carcinoma (NKC) is pervasive, and nonendemic (safe districts), where the keratinizing squamous cell carcinoma (KSCC) is more frequent. Fluctuations in smoking and alcohol consumption amongst genders and geographic regions may clarify the diverse rates and patterns fully observed for KSCC and partially for NKC. Dietary patterns and improvement in disease management can also be accountable for observed trends [18].

2. MicroRNAs discovery

MicroRNAs (miRNAs) are single-stranded, small RNA molecules whose presence was reported for the first time in 1993 in nematode *Caenorhabditis elegans*. A number of biological activities like gene regulation, differentiation, organ formation, cell death, cell proliferation, and stress responses have been reported to be influenced by these miRNAs [19–22]. These miRNAs were found to regulate translation in larval development through an antisense RNA-RNA interaction [19, 23, 24]. 1600 miRNAs of *Homo sapiens* have been reported and recorded by miRBase database in June 2013 [25]. Since 1970s, studies to determine and understand the function and genetics of gene regulation were performed [26]. *C. elegans* was used as a genetic model and tool to facilitate these kinds of ventures. More than 300 mutants of *C. elegans* with many developmental/birth defects and certain behavioral changes were generated by series of groundbreaking experiments by Brenner and Sulston [26, 27]. One of them was characterized by reverberation of specific cell lineages and showing a flaccid and extremely elongated body [27, 28]. The nature of the gene(s) and the exact mechanism underlying such morphological and behavioral changes were unknown at that time. This mutant was considered as the founding member of the miRNA family and was named as lin-4. Lin-4 was reported to influence specific developmental responses in a variety of cell types of *C. elegans* larva [29, 30]. In 1993, cloning of lin-4 locus took about 2 decades after its early description [23]. Lin-4 locus was found unique having characteristics different from other coding genes. It was found employing site-directed mutagenesis, that lin-4 gene encodes a small RNA molecule instead of a protein. Furthermore, the transcripts of lin-4 were of smaller size compared to other genes as the two transcripts identified were of 22 and 60 nucleotides only. To suffice, lin-4 mRNA transcripts were found to be negatively regulating the lin-14 expression, as it possesses antisense complementarity to a number of sites in 3'untranslated region (UTR) to lin-14 gene [23]. Therefore, these discoveries lead to a novel class of small noncoding RNA molecules, which

via antisense-like interaction regulate a number of pathways [31–33]. After 7 years following lin-4 cloning and characterization, a second gene was discovered with similar characters [34]. The study resulted in the isolation of let-7 (21-nt RNA molecule) that was characterized by acting as a heterochronic switch in *C. elegans* development. It was fascinating that the expression of this miRNA was reported in a wide range of animals other than *C. elegans*, including arthropods, mollusks, and mammals [35]. Further studies reported more than 100 different noncoding RNAs (ncRNAs) in a variety of species across the animal phyla [36–38]. A number of important observations were made from these key studies. First, specific small temporal RNAs (stRNAs) express only in few stages of development and are about 22 nt long [38]. Second, cell-type specificity of different stRNAs was determined [38]. For example, miR-1 (a small RNA) exclusively expressed only in cardiac tissues [37]. Presence of homologs of these molecules across a wide range of species is an indication of evolutionary conservation [38]. Abundance and wide existence of these unique molecules represents a novel sequence-specific posttranscriptional gene regulation. So, based on above given account, the small RNAs with similar functions were named as microRNAs (miRNAs) [36–38]. miRNA gene family is increasing day by day since its discovery [39]. Therefore, a number of databases have been generated to cope with the ever-growing list of miRNA genes. The first ever miRNA database is known as "miRBase (http://microrna.sanger.ac.uk/sequences/)" and it has access to clone and register miRNA sequences [40]. The total number of miRNAs is exceeding ten thousands in number up to date.

3. MicroRNAs biology

miRNAs are small ncRNAs of about 19–25 nucleotides and have been discovered recently in all metazoans tested so far [41, 42]. They comprised of a small fraction of expressed genome; however, genes encoding them are present in either introns or exons of coding as well as noncoding genes throughout the genome. miRNAs are involved in regulating number of processes including differentiation, maintenance of homeostasis, migration, programmed cell death, and morphogenesis [42–45]. miRNAs are transcribed with the aid of RNA polymerase II enzyme from the miRNAs genes, which in turn transcribed into primary microRNA transcripts following transformation into pre-miRNAs which are then ultimately results in miRNAs [32, 46–50]. In recent years, a novel class of molecules naming miRNAs has showed a boom. With advancements in genomic technologies and methodologies, miRNAs gene family is rising as new members discovered day by day. In 2005, it was speculated that there are about 1000 miRNAs genes in human genome [51]. However, 2042 mature miRNAs have been reported in miRBase up to date (version 19). miRNAs are characterized by having 21–23 nucleotides in length and are exclusively found in eukaryotes. They show partial complementarity to specific regions in targeted mRNA and hence are involved in gene regulation of 50% of human genome at posttranscriptional level [52]. Each of these noncoding RNAs can have sequence complementary to a large number of transcripts and regulate expression of an enormous number of genes. This can be achieved by multiple mechanisms including an increased mRNA degradation, site-specific cleavage,

and translational inhibition [53]. miRNAs dysregulation has been associated with a number of different types of cancer, their initiation, and proliferation, since the first ever study claiming the association between chronic lymphoid leukemia and miR-15a and miR-16-1 in the year 2002 [54–56]. Therefore, they may prove as potent therapeutic targets for cancers including HNSCC as well as biomarkers for timely diagnosis, prognosis, and recurrence of cancers as well. Two different pathways have been reported for the biogenesis of miRNAs. Usually, a canonical pathway is used for their biogenesis as most of the miRNA genes are intergenic (**Figure 1**). These miRNAs when transcribed possess a local hairpin structure and are named as primary transcripts (pri-miRNA) and have characteristics to that of mRNA, as there is a 5′cap and a 3′poly-A tail. Drosha (a protein complex having nuclease activity) and Pasha (an RNA-binding protein) process these pri-miRNAs to pre-miRNAs having 70-nucleotide stem-loop structure. pre-miRNAs are converted into a double-stranded miRNA/miRNA* duplex by an RNase III endonuclease (Dicer) followed by transportation in cytoplasm by Exportin-5. Further, a helicase destabilizes the duplex miRNA/miRNA* to a mature miRNA and miRNA*. Finally, the mature miRNA then integrates into RNA-induced silencing complex (RISC) also called miRNA ribonucleoprotein complex (miRNP).

The resultant miRNP is involved in RNA interference which is initiated by both siRNA and miRNA. In case of intronic stem-loops-derived miRNA, no Drosha activity is involved for the maturation of miRNAs [32, 57] (**Figure 2**).

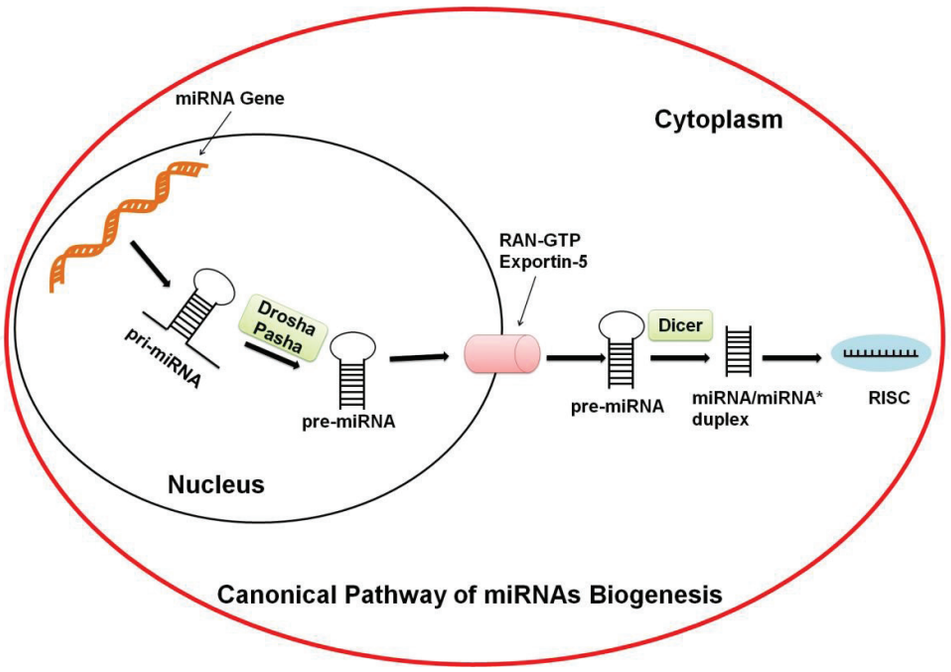

Figure 1. Canonical pathway of miRNAs biogenesis. miRNAs are transcribed into a primary transcript (pri-miRNA) which is processed into pre-miRNAs by Drosha/Pasha complex. After transportation to cytoplasm, these pre-miRNAs are converted into a double-stranded miRNA/miRNA* duplex by Dicer. Further, a helicase destabilizes the duplex miRNA/miRNA* to a mature miRNA and miRNA*. Finally, the mature miRNA then integrates into RISC.

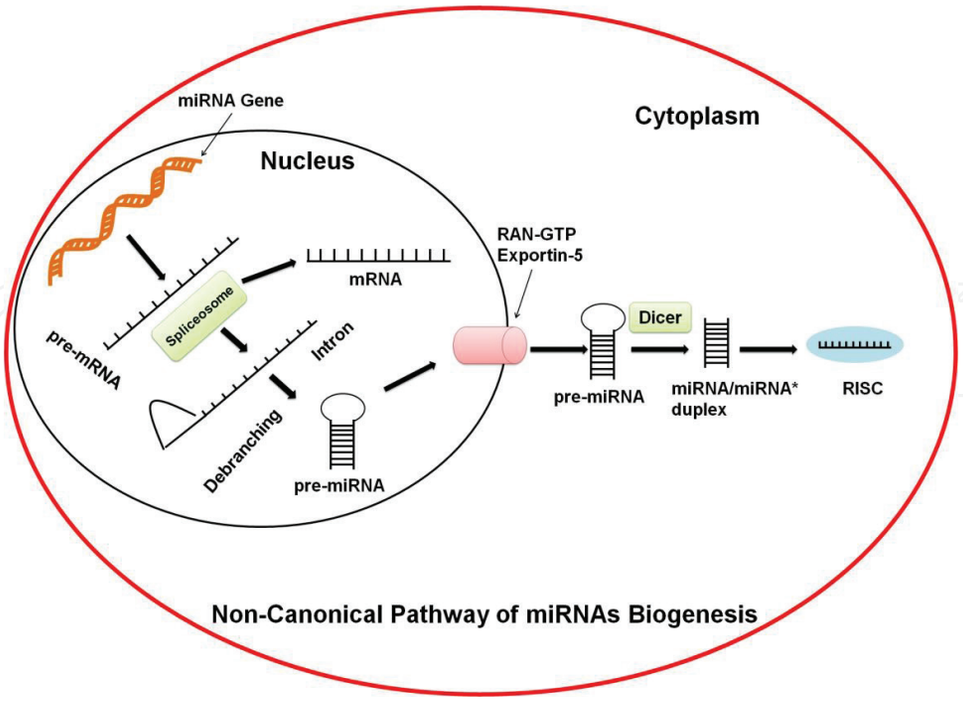

Figure 2. Noncanonical pathway of miRNAs biogenesis. In noncanonical pathway of miRNAs biogenesis, no Drosha activity is involved. Pre-miRNAs are directly produced from the debranching of the introns; and after their transportation, they are processed similar to that produced by canonical pathway.

4. MicroRNAs deregulation in head and neck cancer

The first ever study involving miRNAs in HNC was published in 2005 [58]. miRNAs showed greater cancer-related potential than expected. miRNAs have been associated with all processes of physiological and pathological nature. Expression profiles of miRNAs have reported to be more specific to cancer tissue origin and have greater potential for early diagnosis and provided more information than mRNAs [59, 60]. Association between dysregulation of miRNAs and tumorigenesis in head and neck has been documented previously [61–63]. Many biological and molecular mechanisms cause resistance to the tumors radiotherapy. The principle mechanisms include changes in intracellular pathways that are required in damaging DNA as well as its repair, apoptosis, proliferation, and angiogenesis. The regulation of these perplexing procedures is regularly controlled by microRNAs. miRNAs are short endogenous RNA molecules that posttranscriptionally modulate gene expression. Their impaired expression has been seen in numerous tumors including head and neck cancer. Particular expression patterns of miRNAs have additionally been appeared to anticipate prognosis and therapeutic response in head and neck cancer [64]. Development and progression of different sorts of malignancies in human is attributed to the deregulation of miRNAs. Oncogenic part of miR-214 is proposed in NPC. Bax inhibition initiated by siRNA weakens the advancing impact of miR-214 deregulation on NPC cell apoptosis, recommending that Bax is a downstream effector in miR-214 that is intervened NPC cell proliferation and death. Bax expression level is deregulated in NPC tissues [65]. miR-145 expression in LSCC is downregulated, and its overexpression creates hindrance

in multiplication and relocation of Hep-2 cells through cell cycle arrest and apoptotic induction. SOX-2 is overexpressed in tumor samples and exhibit restricted expression in miR-145 overexpressed Hep-2 cells [14]. miRNAs deregulation plays a key role in HNSCC progression [66]. miR-93-5p (HN2092) and miR-425-5p (HN1957) are top candidates of downregulated miRNAs in primary HNSCC cell cultures and blood plasma of patients [67]. miRNAs deregulation plays a noteworthy part in head and neck/oral cancer [68]. miR-10b and miR-196a, not formerly linked with HNSCC, may show a role in oncogenesis through the deregulation of cell proliferation [69]. Deregulation of miRNA genes (such as miR-138) plays a critical part in HNSCC. While downregulation of miR-138 has been observed in HNSCC and other cancer types often; however, the exact role of miR-138 in tumorigenesis is unknown. Current bioinformatics analyses and *in vitro* and *in vivo* researches have recognized a number of functional targets for miR-138. These include genes that take part in necessary biological processes that are exceptionally pertinent to the onset and proliferation of HNSCC, including cell migration, epithelial to mesenchymal transition, cell cycle progression, DNA damage and repair, senescence, and differentiation [70]. Mechanistic target of rapamycin (mTOR) and Insuline like growth factor 1 receptor (IGF1R) signaling pathways depict that the downregulation of miR-99 family adds to HNSCC tumorigenesis [71]. Meta-analysis of various Gene expression omnibus (GEO) datasets revealed that Uracil DNA glycosylation (UNG), Fucosidase alpha-L-2 plasma (FUCA2), Differentially expressed regulation analysis (DERA), Glia maturation factor beta (GMFB), Transferrin (TF) and Sorting nexin 2 (SNX2) were usually downregulated in HNSCC [72]. miRNAs including miR-21 and miR-31 are progressively deregulated in tongue epithelium [73]. Underexpression of miR-375 might play oncogenic roles in HNSCC [74]. miR-29c is considerably deregulated in

Downregulated miRNAs	Sample used	Assay	Type of cancer	Reference
miR-214	Tissue	Luciferase assay qRT-PCR Western blot	NPC	[66]
miR-145	Tissue	qRT-PCR and Western blot analysis	Laryngeal SCC	[14]
miR-425-5p (HN1957)	Blood plasma	qRT-PCR	HNSCC patients	[68]
miR-93-5p (HN2092)	Blood plasma	qRT-PCR	HNSCC patients	[68]
miR-196a	Tissue	Microarray, RT-PCR	HNSCC	[70]
miR-10b	Tissue	Microarray, RT-PCR	HNSCC	[70]
miR-138	Cell lines	RT-PCR	HNSCC	[71]
miR-99 family	Meta analyses	RT-PCR, microarray	HNSCC	[72]
UNG, FUCA2, DERA, GMFB, TF, and SNX2	Cell lines	RT-PCR	Head and neck squamous cell carcinoma	[73]
miR-21 and miR-31	Saliva, Plasma	q-PCR	Tongue epithelium	[74]
miR-375	Tissue	RT-PCR	HNSCC	[75]

Downregulated miRNAs	Sample used	Assay	Type of cancer	Reference
miR-29c	Cell lines, clinical specimens	Microarray, qRT-PCR	NPC	[76]
miR-9	Cell lines, tissues	qRT-PCR	NPC	[76]
miR-378	Cell lines, tissues	qRT-PCR	NPC	[77]
miR-200 family	Cell lines	Microarray	NPC	[78]
miR-451	Tissue	qRT-PCR	NPC	[79]
MiR-99a	Tissue, cell lines	Luciferase assay	Oral cancer	[80]
		qRT-PCR		
		Western blot		
miR-24	Tissue, cell lines	RT-PCR	Oral cancer	[81]
miR-133a, miR-133b, miR-100, miR-138	Tissues	qRT-PCR	TSCC	[82]
miR-16, miR-125b	Tissues	Microarray	OSCC	[83]
miR-133b, miR-138, miR-137, miR-184	Cell lines	qRT-PCR	OSCC	[84]
miR-138, miR-222	Cell lines	Microarray	TSCC	[85, 86]
miR-342, miR-21	Cell line	Microarray	TSCC	[87]
miR-342, miR-346, miR-373	Cell lines	Microarray	HNSCC	[88]
miR-494	Tissue	Microarray	HNSCC	[62]
miR-375	Tissue	Microarray	HNSCC	[89]
miR-125a, miR-125b	Tissue	Microarray	HNSCC	[90]
miR-133a, miR-205	Tissue	Microarray	HNSCC	[91]
miR-125b, miR-375	Tissue	qRT-PCR	HNSCC	[75]

Table 1. Recently identified miRNAs that undergo deregulation in HNC.

NPC. However, there is dearth of knowledge regarding outcome and molecular mechanisms of action of miR-29c downregulation in development and progression of NPC [75]. Some recent studies indicating miRNAs deregulation in HNC are given in **Table 1**.

5. miRNAs as oncogenes in oral squamous cell carcinoma (OSCC)

A number of miRNAs have been reported to act as oncogenes and were found to be upregulated in OSCC. miR-21 was found to be highly expressed and to regulate several biological

activities in OSCC [68, 92–94]. Presence of an upregulated level of miR-21 in oral premalignant lesions was an indication that variations in miR-21 level may be prior event in OSCC development [95]. miR-21 plays an oncogenic role in the progression of OSCC by promoting cell proliferation [96], antiapoptotic activity [92], invasion [93, 97], and chemoresistance [98] in both *in vitro* and *in vivo* studies.

Oral squamous cell carcinoma (OSCC) is a typical cause of cancer-related death. A number of efforts have been made in investigating new medications and impressive progress in multimodality treatment; however, remedial tolls have not yet been achieved. The trouble of timely detection and more pervasiveness of metastasis associated with OSCC results in poor prognosis. In recent couple of decades, growing information from tumor biology and clinical trials prompted development of ncRNAs prognostic biomarkers that are believed to be promising biomarkers in this regard. miRNAs are one of the most studied ncRNAs in terms of their biogenesis, function, and significance in carcinogenesis [99]. An association between severity of pathogenesis and increment of miR-31 and miR-21 has been reported through staining in 4NQO-induced injury in tongue epithelium. A dynamic rise in the level of *miR-21*, *miR-31*, and *miR-146a* in saliva and plasma samples was noted. miR-31 was the earliest miRNA to be released in the saliva. A rise in plasma level of miR-146a, miR-372, and miR-184 was found and it was prominent at the most progressive lesion state [73]. miRNA deregulation assists in pathogenesis of various disorders, including human tongue squamous cell carcinoma (TSCC), where they act as powerful oncogenes or tumor suppressors. Widespread miRNA profiling in TSCC samples and further *in vitro* and *in vivo* functional studies unveiled their involvement to hidden molecular mechanisms in initiation, development, progression, metastasis, chemoradioresistance, and relapse of TSCC [100]. An upregulated expression of miR-483-5p in OSCC patient sera might be a novel indicative and prognostic biomarker for this ailment [101]. The upregulation of miR-9 was also detected in primary esophageal squamous cell carcinoma (ESCC) tumor tissue. miR-9 promotes cell migration and tumor metastasis, as these processes effectively retarded when expression of miR-9 was deregulated. Furthermore, it was established that miR-9 interacts with the 3′untranslated region of E-cadherin and downregulates its expression, which leads to β-catenin nuclear translocation and consequently upregulates c-myc and CD-44 expression. Moreover, an ESCC miR-9 results in epithelial-mesenchymal transition (EMT) in ESCC, a key occasion in tumor metastasis [102]. Significantly, low level of miR-29b has been reported. However, overexpression of miR-29b repressed the proliferation, migration, invasion, and progression of TSCC cells, and slowed down the cell death. In addition, miR-29b brings about deregulation of Sp1 by targeting the 3′untranslated part that causes upregulation of phosphatase and tensin homolog (PTEN), and subsequently inhibit phosphorylation of Protein kinase B (AKT). Sp1 knockdown in TSCC cell lines reflected the effects of miR-29b overexpression. miR-29b expression is inversely related to Sp1 expression and positively correlated with PTEN. Thus, miR-29b works as a tumor suppressor, and miR-29b/Sp1/PTEN/AKT alliance may speak of a possible therapeutic target for TSCC prevention [91]. MiR-99a deregulation was confirmed in oral cancer cell lines and clinical specimen as well. Ectopic miR-99a expression resulted in repression of oral cancer cell migration and invasion. Myotubularin-related protein 3 (MTMR3) with one evolutionarily

preserved seed locale in the 3'untranslated region was a novel miR-99a target. Draining MTMR3 expression fundamentally diminishes cell proliferation, migration, and invasion. An inverse relation was found among miR-99a and MTMR3 protein in oral cancer lines and clinical patients. miR-99a has been found to suppress oral cancer cell migration and invasion partially through inhibiting MTMR3 expression [103]. Keratinization of tumors and overexpression of miR-21 are major factors responsible for poor prognosis. Interestingly, most of the keratinized tumors were reported to express an elevated miR-21 level [104]. Moreover, an upregulated level of miR-127 and a reduced level of miR-357 have been reported in OSCC [105]. In CD-44 (high) oral CSCs, miR-200s/miR-205s were epigenetically triggered in tumors and their expression was found to be suppressed in the absence of DNA hypermethylation [105]. miRNAs expressions and DNA methylation variations are typical occasion in OSCC, and miR-375, miR-127, miR-137, miR-205, and miR-200 family are promising candidates for future investigations. miR-200/miR-205 downregulation in oral CSCs specify that cell-specific silencing of these miRNAs may enhance tumor expansion and progression [105]. Deregulation of miR-24 was found to be associated with high-grade late stage tumor [92].

6. MicroRNAs in human nasopharyngeal carcinoma (NPC)

Paul Ahlquist's group working at National Cancer Institute (NCI) published the first ever study of global profiling of miRNAs involved in NPC in 2008 [88]. They discovered a number of deregulated miRNAs, using a micro-array-based approach, in laser-capture micro dissected (LCM) 31 NPCs and 10 epithelial samples as control [88]. miRNA downregulation and change in pathways have been involved in NPC which is profoundly invasive and metastatic widespread in Southern China. miR-9 is commonly downregulated in NPC with significant functional consequences. Diminished expression of miR-9 is contrary to clinical stages and denotes the movement from locoregional to metastatic tumors. CpG island hypermethylation adds to inhibition of miR-9 in NPC cell lines and tissues. Ectopic miR-9 expression significantly hinders proliferative, transient, and obtrusive limits of NPC cells, both *in vitro* and *in vivo*. miR-9 strongly reduces expression of CXCR4 in NPC cells. miR-9 works as a tumor-suppressor in NPC, and is interfered by combating CXCR4 expression [106]. NPC is exceptional worldwide, yet profoundly intrusive in later stages. It cannot be identified in normal medical examination at initial stages. Advancement of particular biomarkers ought to spare lives against this sort of ailment. Among them, increased levels of miR-16, miR-21, miR-24, and miR-155 and decreased level of miR-378 have been observed in NPC patients. Plasma miRNA expression and cancer progression are negatively correlated. Blend of miR-16, miR-21, miR-24, miR-378, and miR-155 gives affectability of 87.7% and specificity of 82.0% for marking NPC. Except miR-16, mixture of the rest of four miRNAs gives a similar affectability, however, a somewhat diminished specificity. After treatment, levels of the five miRNAs were reverted to normal levels [88]. Regardless of the fact that enormous miRNAs have been discovered in the previous decade, with the progressions in DNA sequencing, the discovery of some more NPC-related miRNAs is expected. Circulating

miRNAs related with NPC are quite compelling, as they present an exploitable instrumentation for early detection, diagnosis, and staging. A few worldwide profiling depicted the altered miRNA expression in a range of head and neck malignancies. In NPC, downregulation of the tumor silencer miRNAs (miR-29c, miR-9, let-7 family, miR-200 family), and overexpression of oncogenic miRNAs, (miR-18a/b, miR-141, miR-155, miR-214) have been observed [107]. miR-9 is the most concerned miRNA in NPC pathogenesis [108], which works in the direction of various crucial cellular processes, facilitating proliferation, apoptosis, incursion, metastasis, angiogenesis, and EMT [109–111]. Restricted expression of miR-9in NPC is related with phenotypes that are more destructive and lesser survival. miR-9 has been appeared to work as a tumor silencer in NPC by focusing on chemokine (CXC theme) receptor 4 (CXCR4) to repress cell multiplication, relocation, and invasion [87]. Similarly, miR-9 directs innate response in NPC through control of various genes that are activated by interferon, and in addition major histocompatibility complex (MHC) class I members [112]. Imperatively, the capacity of miR-9 to work as an individual prognostic biomarker for NPC metastasis has additionally been illustrated, whereas miR-9 expression is related with decreased expansion, movement, and intrusion in NPC cells, and low-levels of miR-9 expression are associated with last stage [106]. Correspondingly, miR-200 family is deregulated in NPC, bringing about expanded cell development, relocation, and intrusion of NPC cells according to suppression of the putative miR-200 targets zinc finger E-box restricting homeobox 2 (ZEB2), beta 1 (CTNNB1), and catenin (cadherin-related protein) bringing about expanded NPC cell development, movement, and invasion. Moreover, diminished miR-200a expression is related with initiating epithelial-mesenchymal transition [89]. Various miRNAs have been reported as tumor silencers/suppressors in NPC, including miR-34c, miR-451, miR-98, miR-216b, miR-375, and miR-26a. miR-375 has been accounted for a potential tumor silencer in NPC, working through restraint of the oncogenic protein metadherin (MTDH). Strangely, NPC cases with MTDH overexpression showed an expanded danger of recurrence of disease [113]. Low miR-451 expression was related with diminished survival in NPC patients, and has been appeared to work by expanding cell development and invasion by focusing on macrophage migration inhibitory factor (MIF) in NPC cells [114]. miR-26a, miR-98, and miR-101 also have been accounted to work as tumor suppressors in NPC [98]. Underexpression of these miRNAs in NPC gives clues about derepression of Enhancer of zeste homolog 2 (EZH2), instigating loss of suppression of targets of EZH2 regulation, comprising cyclins D3, c-Myc, and E2, and cyclin-dependent kinase 6 (CDK6) and CDK4 [115, 116]. Finally, miR-216b has been appeared to increase NPC growth and incursion by pursuing Kirstan rat sarcoma (K-RAS) [117]; on the other hand, miR-34c has been revealed to stifle growth and metastasis by focusing on the proto-oncogene MET [118], both of which are ominously downregulated in primary NPC tissues. In NPC, miR-18a is profoundly overexpressed [119], which results in direct hang-up of the miRNA biogenesis regulatory protein Dicer1, leading to the downregulation of miRNA in NPC [119]. Likewise, miR-18b is overexpressed in NPC, accompanying malady advancement and poor outcome. miR-18b functions to inhibit connective tissue growth factor (CTGF) and thereby augmenting cellular proliferation [120]. Over-articulation of miR-141 in NPC has been connected with more cell growth, relocation, and invasion, and also with loss of cell cycle

control and decreased apoptosis. This regulation is thought to happen through downregulation of the supposed target gene phosphatase and tensin homolog (PTEN), bromo-domain 3 (BRD3), and ubiquitin-associated protein 1 (UBAP1) in NPC. Moreover, expression of miR-141 is structured by the oncogenes short palate, lung, nasal epithelium clone 1 (SPLUNC1), and c-Myc [120]. Expression of miR-155 fortifies proliferation, migration, and incursion by regulation of target genes Jumonji Domain 1A (JMJD1A) and BTB and CNC homology 1 (BACH1), and its expression has been strongly related with tumor stage and patient endurance. Interestingly, regulation of miR-155 occurs through the Epstein–Barr virus (EBV)-encoded Latent membrane protein (LMP1) and LMP2A proteins [121]. Furthermore, miR-144 is overexpressed in NPC and inhibits PTEN expression, and brings about expanded cell proliferation, invasion, and metastasis [122]. miR-214 has been firmly connected with augmented metastasis in NPC, both in cell lines and primary human samples, functioning at least in part via inhibition of the tumor suppressor lactotransferrin (LTF) [123]. Besides, miR-214 has been appeared to upgrade proliferation and promote an antiapoptotic phenotype in NPC cells [124]. In addition to miR-144, miR-155, and miR-214, various other miRNAs that are overexpressed in NPC also contribute considerably to enhance the metastatic phenotype of NPC. miR-30a has been demonstrated both *in vitro* and *in vivo* to build metastasis and intrusion by hindering E-cadherin activity [125]. Moreover, expression of miR-149 was lifted profoundly in metastatic NPC cells, adding increased migration, invasion, and epithelial-mesenchymal phenotypes over E-cadherin inhibition [126]. miR-93 has been accounted to inhibit transforming growth factor-β receptor II (TGFβRII) [127] and disabled homolog-2 (DAB2) [128], in this way directing tumor cell growth incursion and metastasis. Finally, miR-504 is found to be involved as an oncogenic miRNA in NPC, working to specifically target nuclear respiratory factor 1 (NRF1), whereas expanded expression connected with poor reaction to radiation treatment [129].

7. miRNAs as prognostic indicators

Despite significant improvement in multimodal treatment, the prognosis of advanced HNC is quite poor. Recent studies are promising regarding the potential role of miRNAs as prognostic indicators. Downregulation of Let-7 (a family of tumor suppressing miRNAs) has been reported in HNSCCs by many researchers [74, 92, 95, 103, 130–138]. Similarly, underexpression of miR-146a, miR-155, and Let-7 has been correlated with the progression of cancer [138]. Moreover, a decreased level of Let-7 miRNA in nasopharyngeal carcinoma cells was also suggestive of regulating the proliferation of carcinoma cells via c-MYC downregulation [139]. Furthermore, role of Let-7 in the Kirsten rat sarcoma (KRAS) regulation has been demonstrated recently by some studies on nonsmall cell lung cancer [140]. A variant allele in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) 3′untranslated region (KRAS-LCS6) has been demonstrated to be involved in high expression of KRAS and decreased levels of Let-7. Presence of KRAS-LCS6 variant has been reported in Squamous cell head and neck cancer (SCHNC) and was correlated with poor prognosis [130]. TP53, a tumor suppressor gene, encodes a protein product that is

involved in cell cycle regulation. It is a major change involved in cancer induction with a remarkable frequency of 53% as reported by Poeta et al. in a hefty cohort of SCHNC. The ratio of TP53 mutations were 75 and 56.7% in patients suffering from hypopharynx and larynx tumor, respectively. Furthermore, a strong association was described between TP53 mutations in SCHNC and high risk of recurrence and poor survival [141]. Ganci et al. has previously reported a strong link among 49 miRNAs and TP53 status. Among them, a more specific correlation of a subset of 12 miRNAs was correlated with a brief recurrence free-survival while four of these were correlated with comparatively lower cancer-specific survival [142]. Expression of some particular miRNAs, such as miR-375 and miR-210, has been correlated with the outcome of SCHNC patients. A low expression of miR-375 results in poor survival and distant metastases while high expression of miR-210 results in locoregional recurrence [143, 144].

8. miRNAs as biomarkers

Diagnosis of HNSCC more frequently occurs in advanced stage when it metastases to regional lymph nodes. Moreover, there is a high risk of recurrence even in patients with a combination of different therapeutic approaches. Therefore, for an early diagnosis, the most important goal would be the detection of biomarkers. Recently, some miRNAs have been discovered to fulfill the goal of diagnosis. In fact, miRNAs are found circulated stably in different body fluids, i.e., urine, blood, saliva, as well as in breath. Hence, these miRNAs can be assessed easily with noninvasive methods.

miRNAs can be utilized as biomarkers or novel therapeutic targets. Further investigation is needed to test its utilization [66]. Circulating miRNAs (miR-425-5p, miR-93-5p) are easily approachable and have turned out to be valuable prognostic markers in cancer patients. The prognostic worth of this exciting perception requires affirmation using an independent patient cohort that includes clinical follow-up data. Changes of miRNAs succeeding radiochemotherapy in the blood plasma are related with the tumor response to therapy, and they might signify novel biomarkers for therapy monitoring [67]. The investigations of microRNA modifications in HNSCC are a fundamental stride to the mechanistic comprehension of tumorigenesis and could prompt the disclosure of clinically pertinent biomarkers [69]. For NPC diagnosis, plasma miRNAs expression proves to be a helpful biomarker [88]. Pathway improvement analysis of these four miRNAs (miR-34c, miR-140, miR-154, and miR-449b) sign posted a role in cell cycle regulation, highlighting a possibly important role for markers of cell cycle enactment as prognostic indicators in NPC [145]. miR-200b expression is mostly connected with distant metastasis, while miR-155 associated with local recurrence. miR-155 and miR-146a were recognized as surrogate markers for tumor-invading lymphocytes in HNSCC [146]. miR-21 expression could be an imperative tool for treatment planning and prognostic predictor for HNSCC patients [147]. Genetic variants of miR-146a and miR-1269b are biomarkers for improvement of oral premalignant lesions (OPLs) and oral squamous cell carcinoma (OPSCC) [148]. hsa-miR-375-3p appears to be a comparatively promising diagnostic marker

miRNA biomarker	Type of cancer	References
miR-34c, miR-140, miR-154, and miR-449b	NPC	[166]
miR-155 and miR-146a	HNSCC	[167]
miR-155		
miR-21	HNSCC	[168]
miR-146a and miR-1269b	Oral premalignant lesions (OPLs) and oral squamous cell carcinoma (OPSCC)	[169]
hsa-miR-375-3p	HNSCC	[170]
miR-182	HNSCC	[171]
miR-30a	NPC	[172]
miR-148a and miR-375	LSCC	[173]

Table 2. A list of miRNAs identified as potent biomarker of HNC.

in HNSCC but is not appropriate for prognosis of patients [149]. Overexpression of TP53 mutation-associated miR-182 may contribute to proliferation and migration of tumour cell in HNSCC, hence suggest a possible biomarker for prognosis of tumour recurrence [150]. miR-30a is a possible biomarker for metastasis in NPC patients [151]. miR-148a and miR-375 are significantly upregulated during LSCC and are conceivable biomarkers for early diagnosis of LSCC [152]. A list of miRNAs is given below which has been identified as potential biomarker by a number of researchers in HNC (**Table 2**).

9. Novel therapeutic targets

miRNAs are key therapeutic agents (depends upon the type of mRNA affected by them) in addition to their prognostic and diagnostic value [21]. miRNAs are efficient molecules to be targeted as they regulate a number of biological activities by interacting with numerous other molecules [53]. Use of synthetically designed "miRNAs sponges" [153], miR mimics (agomiRs) [154], miR antagonists ("antagomiRs") [155, 156], and miR inhibitors (antimiRNAs oligonucleotides) [92] is an innovative strategy to modulate oncogenic and tumor-suppressive pathways. MicroRNAs regulate each step of cell cycle and unraveling their altered expression may prove fruitful in designing new drugs and can open treatment regimes. One piece of evidence is a previous study abduction of miR-122, which has been observed in a novel therapy for HCV patients by Miravirsen (first microRNA targeted drug) in clinical phase2a. This therapy resulted in diminution of HCV RNA levels in a dose-dependent manner [157]. This research has raised a lot of hope for employing miRNAs therapies in number of malignancies despite the disparity of the diseases from cancer. Some other miRNAs

therapies are in preclinical and clinical phase 1. These approaches are anxiously awaited to be reported beyond the mere documentations. Few miRNAs, though not verified clinically, are very good applicants for being named for these therapies. Apoptotic pathway is one of the most significant candidates for novel anticancer therapies, as the neoplastic cells usually lose the ability to undergo programmed cell death. Therefore, any powerful proapoptotic agent may directly or indirectly reduce cancer progression by enhancing the apoptosis. *miR-99a* mimics have been shown to decrease cell proliferation by inducing apoptosis in tongue SCC cell line [158]. Moreover, miR-31-3p inhibitor in oral leukoplakia cell lines resulted in decreased cell death [159]. Furthermore, restoration of miR-100 resulted in inhibition of cell migration and proliferation by increasing the apoptosis in HNSCC cell lines [71]. Similarly, cancer cells showed a pronounced cell cycle arrest and enhanced cell death upon transfection with miR-1e [160]. On the other hand, a repression of apoptosis was observed in classical oncogenic *miR-21* [161]. These are only a few well-known examples. A large number of other functionally investigated miRNAs in HNSCCs are directly or indirectly associated with apoptotic pathways promise an era of new and more potent therapeutic factors. miRNAs are utilized in another way in treating the HNSCC patients, as they are capable of highlighting the resistance to either chemotherapy or radiotherapy in patients of HNSCC patients. In addition to it, recently, miRNAs have also been reported to modulate the radiosensitivity and chemoresistance. For instance, let-7 results in inhibition of cancer progression by reduction in cancer-proneness cells and repressing chemoresistance [162]. Similarly, miR-21 in HA/CD44-activated head and neck cancer cells may prove a significant drug target to overcome chemoresistance and apoptosis as it is involved in regulation of Nanog/Stat-3 signaling pathway [111]. Furthermore, transfection of pre-miR-98 in HNSCC cell lines led to an enhanced resistance to doxorubicin and cisplatin by downregulating the *HMGA2* expression [163].

Nevertheless, for an efficient and target specific miRNAs-based drug delivery system, there lies enormous challenges [22, 53]. To access the targeted sites, therapeutic RNA must travel across the plasma membrane to enter in cytoplasm by leaving the circulatory system and avoiding endosomal vesicles to entering in the cell [53]. Moreover, nonconjugated therapeutic RNA molecules are either cleared by the kidney (<50kDa) or by the immune cells (7-20kDa) [53]. Levels of mature miRNAs may be modulated or reduced by delivery of synthetic double-stranded hairpin exogenously by complexing with lipids or proteins. Delivery of miR-34a may prove effective in HNSCC cells as suppression of cellular proliferation and apoptosis was observed in two cancer cell lines (colon) and experimental lung metastasis of murine melanoma upon miR-34a delivery [164, 165]. Use of unmodified dsRNAs in environments, where local administration is possible, is of limited value *in vivo* as they are likely to be degraded by nucleases [166]. A high expression of these miRNAs from well-defined transcription start and termination sites can be achieved using a viral vector with Pol III promoters for stable miR reintroduction; however, they are not cell-specific [53, 165, 167]. In contrast, for a tissue-specific approach or for ectopic expression of miRNAs, RNA Pol II promoters may be used for pri-miRNAs expression [168]. For an effective reintroduction of dysregulated miRNAs, later techniques may be utilized in HNSCC, e.g., miR-375 [132]. However, use of a viral system for reintroduction of miRNAs possesses a number of risks [53]. One of the major risks is that the delivered molecules integrate to an unpredictable site of host DNA and may activate proto-oncogene and

results in insertional mutagenesis [53]. These methods also have certain limitations as insertion of retroviral vectors is limited only to actively dividing cells and use of other units, like adenoviral vectors, may activate a severe immune response in host body [53, 169]. AntagomiRs (recently identified chemically engineered oligonucleotides) are commonly utilized today for silencing of host miRNAs [53, 156]. These AntagomiRs reverted the effects of upregulation of miRNAs in HNSCC, i.e., decreased cell viability and enhanced programmed cell death was observed in KB cells overexpressing miRNA-155 upon introduction of antagomir-155 in nude mice [155]. Moreover, to boost the efficient delivery of these antagomiR, they are delivered intravenously by complexing with a recently identified molecule the interfering nanoparticle (iNOP) [170]. Systemic delivery of iNop complexed with antimir-122 in mice did not initiate any immune response and successfully silenced the miRNA-122 with long lasting effects [170]. Chemically modified antimiRNAs oligonucleotides (AMOs) that are highly specific and efficient in binding to targeted RNA are another choice for miRNAs silencing but they do not have any specific delivery system [92, 164]. An enhanced apoptosis, limited survival, and proliferation were observed in tongue SCC cell lines upon inhibition of miRNA-21 with AMO [92]. Moreover, a pronounced apoptosis and decreased cell proliferation was observed in nude mice upon repeated injections of miR-21-AMO which ultimately resulting in tumor suppression [92]. In HNSCC, miRNAs sponges or miRNAs masks can also be used to inhibit oncomiRNAs. miRNAs sponges block specific miRNAs attachment to their targets by expressing an mRNA having a number of tandem-binding sites for entire family of targeted miRNAs, thus blocking their effects [53, 153]. On the other hand, miRNAs-masking antisense oligonucleotides technology shows its effect by destabilizing the association between specific miR-mRNA pairs, as it comprised of complementary antisense oligonucleotides to miRNAs interacting site in the 3'UTR of a targeted mRNA [53, 167]. This methodology has advantage over others as there are few off-target/undesirable effects and may be helpful in cancer therapy as multiple pathways are targeted there simultaneously [53]. Furthermore, miRNAs may enhance sensitivity to radiotherapy [168]. In addition to it, some epigenetic drugs may also revert irregular methylation or acetylation of a tumoral phenotype by restoration of tumor-suppressive miRNAs expression [53]. Most of above-mentioned techniques are in experimental phases. Our future approach should aim to aberrant miRNAs networks, as multiple miRNAs along with multiple miRNAs-transcriptome interactions are involved in cancer pathogenesis. To achieve this aim, miRNAs biogenesis machinery or regulatorypathways must be targeted [53, 169]. However, exploration of the full potential ofthese drugs is highly recommended.

10. Future challenges

For last 3 decades, dysregulation of tumor suppressor genes and protein-coding oncogenes were thought to be involved in cancer. However, discovery of noncoding genes, i.e., miRNAs raised a question on the notion that this mechanism is solely responsible for cancer. To unveil the involvement of miRNAs in cancer, massive efforts have been made but still enormous challenges lie ahead. Detection of the exact pathways and genes regulated by these miRNAs will be of prime importance. A better understanding of the wide-ranging effects of these

novel molecules will equip the researchers in the selection of specific pathways for treatment of cancers. Our understanding of miRNAs involvement in HNC is in its infancy. However, studies have been confirmed the dysregulation of a number of miRNAs in both benign and malignant HNC. There are real prospects that miRNAs in near future may be used as prognostic and diagnostics of HNC to improve its treatment strategies. Growing evidences make these miRNAs viable targets for the development of new and better anticancer therapies. However, before translation to be carried out in clinical settings, a more clear insight of efficacy and "off-target" effects of miRNAs is necessarily suggested. It is quite fascinating that recently a number of miRNAs including miR-21, miR-155, and let-7b have been nominated as key players of human carcinogenesis including head and neck tumors. The discovery of miRNAs heralds a complete new paradigm in the understanding of exact molecular pathways involved in HNC development. More detailed studies are required for better understanding and therapeutic targets to treat HNC.

However, a number of efforts have been made but truly comprehensive profiling of miRNAs is still needed in HNSCC. Even, technology utilized for profiling also needs to be improved. Use of Polymerase chain reaction (PCR) and microarrays is a common practice for profiling but for a better picture and more deep insight next-generation sequencing (NGS), cross-platform analysis and new approaches like NanoString nCounter system must be applied. Functional studies are growing, highlighting the potentially targetable miRNAs and some related pathways. Tumor cells survival and their development have been reported to be affected by the forced expression or even the inhibition of a number of miRNAs *in vitro*, representing a new class of novel therapeutic targets [158, 171, 172]. To deliver therapeutic miRNAs, delivery of pre-miR-107 and siRNA nanoparticles proves to be an effective strategy [173, 174]. Therefore, the future of tumor biology seems quite bright.

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